Summary
Intrafollicular luteinization stimulator was shown to be secreted by granulosa cells in culture with stimulatory effects on differentiation of immature granulosa cells. The purpose of this study was to evaluate the role of calcium ions in luteinization stimulator-enhanced luteinization process of granulosa cells. We examined the direct effect of ionophore A23187, voltage-sensitive Ca\(^{2+}\)-channel blockers verapamil, nimodipine, nifedipine, niludipine and calmodulin antagonist trifluoroperazine on progesterone and cGMP levels in 3-day culture of small granulosa cells. It was shown that the dihydropyridine derivatives of calcium blocker drugs (nimodipine, nifedipine, niludipine) and calmodulin antagonist (trifluoroperazine) in the micromolar range, significantly suppressed FSH-induced progesterone synthesis and cGMP accumulation in granulosa cells. On the contrary, phenylalkylamine calcium blocker verapamil and calcium ionophore A23187 had different effects on both processes. Calcium ionophore A23187 markedly enhanced cGMP formation, but simultaneously inhibited the FSH-induced progesterone synthesis. Verapamil at lower concentrations (10 \(\mu\)M) stimulated and at higher concentrations (50 \(\mu\)M) inhibited the formation of cGMP. To evaluate the role of extra- and intracellular calcium in luteinization stimulator-enhanced progesterone production by small granulosa cells, the effects of indicative agents on stimulatory activity of follicular fluid from large follicles, granulosa cells-conditioned media and granulosa cell extracts were tested. While verapamil is shown to be a less potent modulator, administration of other calcium antagonists as well as ionophore A23187 caused a significant decrease in stimulatory action of follicular fluid from large follicles, granulosa cells-conditioned media and extracts. These findings indicate that the stimulatory action of luteinization stimulator depends on the transport of calcium ions through voltage-sensitive calcium channels and is modulated by alteration of intracellular calcium levels.

Key words
Follicular fluid – Calcium blockers – Progesterone – cGMP – Granulosa cells

Introduction
Calcium ions belong to intracellular second messengers which are involved either individually or together with the other signalling systems, cyclic nucleotides and diacylglycerol, in regulation of mammalian cell function (for review see Davis et al. 1992). Recent studies indicate that calcium participates in steroid hormone production by isolated ovarian cells. Short-term incubation of granulosa cells, isolated from porcine preovulatory follicles treated by LH in Ca\(^{2+}\)-deficient cultures, suppressed cAMP and progesterone production (Veldhuis and Klase 1982) and selectively impeded the capacity of 8-bromo-cAMP to stimulate pregnenolone synthesis from the endogenous sterol substrate (Veldhuis et al. 1984). Calcium antagonist verapamil and TMB-8 suppressed steroidogenic effect of LH in porcine and hen granulosa cells (Veldhuis and Klase 1982, Asem and Hertelendy 1986). Eckstein et al. (1986) demonstrated that the use of verapamil and calmodulin antagonist trifluoroperazine (TFP) inhibited stimulatory action of GnRH and LH on progesterone production in rat granulosa cells. In hen granulosa cells, TFP suppressed LH-induced cAMP synthesis, while calcium ionophores (ionomycin and A23187) potentiated cAMP responses to LH (Jamaluddin et al. 1992). A23187 has been shown to affect LH-induced progesterone production.
in porcine (Veldhuis and Klase 1982) and rat (Carnegie and Tsang 1984) granulosa cells, in bovine luteal cells (Benhaim et al. 1990) as well as cAMP and testosterone production in goldfish preovulatory ovarian follicles (Van Der Kraak 1992).

The aim of the present study was to investigate the role of calcium ions in the regulation of immature granulosa cell differentiation induced by FSH and intraovarian lutenization stimulator (LS). LS, a non-steroidal compound produced into follicular fluid during follicular development, modulates the regulatory effect of gonadotropins on granulosa cells (Channing et al. 1982). As was reported previously, LS enhanced progesterone production and LH/hCG receptor induction in immature granulosa cells (Kolena and Channing 1985). The stimulatory factor was partially purified from follicular fluid of large follicles (LFF) with a molecular weight about 28 000 (Kolena and Channing 1985). LS activity was further demonstrated in granulosa cell cultures; the factor is produced preferentially by granulosa cells isolated from preovulatory follicles (Seboková et al. 1987) and its secretion is hormonally regulated (Danišová and Kolena 1992). However, the specific mechanism subserving the stimulatory effects of LS on granulosa cell differentiation is not known. The absence of alterations in cAMP synthesis by granulosa cells treated with LFF (Kolena et al. 1993) allow us to suggest that LS probably does not operate through the adenylate cyclase signalling system. LFF was shown to enhance cGMP levels in granulosa cell cultures (Kolena et al. 1993). However, the physiological relationship between cGMP synthesis and steroid production remains to be further investigated. In order to evaluate the possible role of calcium in mechanism of LS action, we have tested the effect of calcium ionophore A23187 and various calcium and calmodulin antagonists on the stimulatory activity of LFF, granulosa cell-conditioned media and cell extracts.

Material and Methods

Hormones and chemicals
Porcine FSH (FSH-F-1) was generously supplied by NIAMDD, NIH (Bethesda, Maryland, USA). Insulin (I), thyroxin (T₄), ionophore A23187 (A), verapamil (VP) and Medium 199 with Earle’s salts were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Nimodipine (NM), niludipine (NL) and nifedipine (NF) were obtained from Bayer (Wuppertal, FRG), trifluoroperazine (TFP) from Merck (Darmstadt, FRG). Fungizone (amphotericin B) was purchased from Serva (Heidelberg, FRG), penicillin, streptomycin and glutamine from Spofa (Prague, Czech Republic). [¹²⁵I]-Progestrone was from the Institute of Radioecology and Exploitation of Nuclear Technique (Košice, Slovakia). Tissue culture 24-wells plates were obtained from Flow Labs (Rockville, MD, USA). Stock solutions of A23187, nimodipine, nifedipine, niludipine and trifluoroperazine were prepared in ethanol, verapamil was dissolved in H₂O. Reagents were kept shielded from light and stored at −20 °C.

Cell cultures
Porcine ovaries from approximately 6-month-old animals were obtained at a local slaughterhouse and transported to the laboratory in physiological saline plus antibiotics on ice. Granulosa cells were harvested from small (1–2 mm) ovarian follicles (SGC) as previously described (Kolena and Channing 1972). The cells were washed twice in serum-free media and incubated at a density 1–12 x 10⁶ viable cells per well. Cell viability was estimated by counting in a haemocytometer with 0.06 % trypan blue. Granulosa cells were cultured at 37 °C in an atmosphere of 5 % CO₂ and 95 % air in tissue culture multiwell plates containing Medium 199 with Earle’s salts and Hepes buffer (250 mmol.l⁻¹) in a volume of 0.5 ml for 3 days. The culture medium was supplemented with pig serum (10 %), L-glutamine (1 mmol.l⁻¹), porcine FSH (1 mU.ml⁻¹), thyroxin (0.1 mmol.l⁻¹), insulin (1 mU.ml⁻¹) and antibiotics fungizone (0.25 µg.ml⁻¹), penicillin (100 U.ml⁻¹) and streptomycin (100 µg.ml⁻¹). The cells were cultured for 3 days in the presence of specific agents. At the end of the incubation period, the media were collected and stored frozen at −20 °C until assayed for progesterone and cGMP.

Follicular fluid was collected from 6–12 mm large follicles (LFF) by aspiration and centrifugation at 1000xg for 15 min. Granulosa cells-conditioned media (CM) were obtained from cultures of granulosa cells isolated from large porcine follicles (LGC). LGC were cultured at the same concentrations and under the same experimental conditions as described above for the granulosa cell culture procedure. Cell extracts were obtained according to Makris et al. (1983). The homogenate prepared from freshly collected LGC (2.2–2.6 x 10⁶ cells per ml) was centrifuged at 105 000xg for 1 h.

Progesterone levels in culture media were quantified by [¹²⁵I]-progesterone radioimmunoassay using specific antiserum against 11-OH-progesterone succinyl-BSA (kindly donated by Dr. Tománek, Research Institute of Animal Production, Prague, Czech Republic) without extraction (Kolena and Channing 1985). cGMP content was measured by radioimmunoassay after acetylation of the samples by using a [¹²⁵I]-cGMP RIA kit (Institute for Research, Production and Application of Radioisotopes, Prague, Czech Republic).

The results from experiments (run in quadruplicates) were expressed as means ± S.E.M. Statistical significance was analyzed by Student’s t-test.
Fig. 1
Effect of ionophore A23187 (A), Ca^{2+}-channel blockers verapamil (VP) and nimodipine (NM) and calcium antagonists nifedipine (NF), niludipine (NL) and trifluoroperazine (TFP) on progesterone production by immature granulosa cells (SGC). SGC (1.1 x 10^7 per ml) were cultured for 3 days in the medium with FSH+I+T_4 (C) or in the same medium with A, VP, NM, NF, NL and TFP. The data are means ± S.E.M. (n=4).

Fig. 2
Influence of sodium azide (NaN_3), ionophore A23187 (A), verapamil (VP), nimodipine (NM), nifedipine (NF), niludipine (NL) and trifluoroperazine (TFP) on cGMP synthesis by SGC. SGC were cultured as described in Fig. 1. Results are representative of 3 experiments.
Results

It was observed in preliminary experiments that chelation of extracellular Ca$^{2+}$ by EGTA significantly decreased progesterone production in immature granulosa cell cultures stimulated by FSH and LFF (data not shown). In order to evaluate the role of extracellular calcium, we used calcium ionophore A23187 and some inhibitors of transmembrane calcium transport. A23187 in the used concentration 2 µM significantly (P< 0.001) decreased FSH-induced progesterone synthesis by granulosa cells (Fig. 1). Incubation of granulosa cells with the voltage-dependent Ca$^{2+}$-channel blocker verapamil (10 and 50 µM) resulted in a significant (P< 0.001) inhibition of progesterone secretion. Administration of dihydropyridine calcium channel blockers nimodipine, nifedipine and niludipine (10 and 100 µM) to the granulosa cell culture also significantly suppressed (P<0.001) the production of progesterone.

To examine the possible role of calmodulin in mediating the FSH action in immature granulosa cells, the culture was exposed to the calmodulin antagonist trifluoroperazine (10 and 50 µM). As shown in Fig. 1, trifluoroperazine significantly (P<0.001) inhibited the synthesis of progesterone.

In contrast to the inhibitory effect of A23187 on progesterone production, ionophore significantly (P<0.005) stimulated cGMP synthesis by granulosa cells (Fig. 2). A similar stimulatory effect was achieved by verapamil in a lower dose (10 µM), while the higher concentration of verapamil (50 µM) did not alter cGMP levels. Maximal cGMP responses were observed after the cultivation of granulosa cells in the presence of sodium azide, a non-specific stimulator of guanylate cyclase activity. Under identical conditions, nimodipine, nifedipine, niludipine and trifluoroperazine significantly decreased cGMP accumulation at both concentrations (P<0.005).

![Fig. 3](image)

Suppression of follicular fluid-stimulated progesterone release by SGC in the presence of inhibitors. SGC (1 x 10^7 per ml) were cultured 3 days only with FSH+1+T4 as a control (C) or the same with 25% follicular fluid (LFF) and LFF plus A23187 (A), verapamil (VP), nimodipine (NM), nifedipine (NF), niludipine (NL) and trifluoroperazine (TFP). Values represent means ± S.E.M. of 4 estimations repeated four times with similar results.
In the next series of experiments, we studied the effect of ionophore A23187 and Ca\textsuperscript{2+}-antagonists on the follicular fluid-stimulated steroidogenic response of granulosa cells (Fig. 3). Follicular fluid used in the 25\% concentration stimulated progesterone production by granulosa cells to 3-fold above the control level (P<0.001). Administration of A23187 suppressed (P<0.001) this stimulatory activity of follicular fluid. The same results were obtained by using calcium antagonists nimodipine, nifedipine, niludipine and trifluoroperazine at both concentrations tested (P<0.001). Verapamil impaired the stimulatory effect of follicular fluid only at a higher concentration (50 \mu M; P<0.005).

We have shown in previous studies that granulosa cells in culture are able to secrete stimulatory activity which meets the criteria for luteinization factor found in follicular fluid from preovulatory follicles (Šeböková et al. 1987, Danišová and Kolena 1992). Therefore, we compared the effect of granulosa cells-conditioned media (in the 50 \% concentration) on steroidogenesis in the presence of calcium ionophore and antagonists (Fig. 4). Conditioned media obtained from 3-day cultures of granulosa cells from preovulatory follicles further enhanced progesterone secretion by immature granulosa cells in comparison to FSH+I+T\textsubscript{4} controls (P<0.001). Fig. 4 shows the inhibitory effect of 2 \mu M A23187 (P<0.005), 50 \mu M verapamil (P<0.02), 10 \mu M and 100 \mu M nimodipine (P<0.005; P<0.001) on progesterone synthesis by granulosa cells stimulated with conditioned media. The inhibitory effects of 10 and 100 \mu M nifedipine (P<0.005), 10 and 100 \mu M niludipine (P<0.005; P<0.001) and 50 \mu M trifluoroperazine (P<0.005) on progesterone production enhanced by conditioned media are also illustrated.

Finally, we have also investigated the effect of ionophore A23187 and calcium antagonists on the activity of granulosa cell extracts. Freshly prepared extracts of granulosa cells isolated from preovulatory follicles used in 50 \% concentration stimulated (P<0.001) progesterone production by immature granulosa cells, similarly as the effect of conditioned media. As depicted in Fig. 5, significant suppression of progesterone synthesis stimulated by cell extracts were observed in these studies during cultivation of granulosa cells either in the presence of ionophore A23187 (P<0.001), channel blockers verapamil (10 and 50 \mu M; P<0.05), nimodipine (10 and 100 \mu M; P<0.02 and P<0.001), nifedipine (10 and 100 \mu M; P<0.001), niludipine (10 and 100 \mu M; P<0.001) or the calmodulin antagonist trifluoroperazine (10 and 50 \mu M; P<0.001).
Fig. 5
Progesterone synthesis by SGC in response to addition of granulosa cell extracts (Ex), A23187 (A), verapamil (VP), nimodipine (NM), nifedipine (NF), niludipine (NL) and trifluoroperazine (TFP). Concentration of extracts in culture medium was 50%. For details see legend to Fig. 1.

Discussion

The present study indicates that calcium ions modulate the steroidogenic response of immature granulosa cells in culture induced by FSH and intraovarian luteinization stimulator (LS). The LS action was tested by applying follicular fluid from large follicles (LFF), granulosa cell-conditioned media and granulosa cell extracts in which LS activity had previously been detected (Channing et al. 1982, Sebokova et al. 1987). The objective of this study was to determine whether the modulation of extracellular Ca\(^{2+}\) movements across the plasma membrane would influence the steroidogenic responses of granulosa cells to the stimuli. To achieve this effect, we selected calcium and calmodulin antagonists and calcium-ionophore, which preferentially block and/or enhance Ca\(^{2+}\) transport across biological membranes.

Progesterone production by granulosa cells stimulated by FSH and LS was suppressed after adding Ca\(^{2+}\)-channel blockers. Our results are consistent with the observations reported for porcine (Veldhuis and Klase 1982, Veldhuis et al. 1984), hen (Asem and Hertelendy 1986) and rat (Eckstein et al. 1986) granulosa cells. In these studies, inhibition of LH-stimulated cAMP and progesterone production was demonstrated in the presence of verapamil. In calcium-deficient incubations, calcium ions specifically modulated LH stimulation of pregnenolone biosynthesis from endogenous cholesterol substrate, but did not influence basal pregnenolone production or progesterone production from exogenously supplied cholesterol substrate (Veldhuis et al. 1984). It is suggested that extracellular calcium regulates steroidogenesis at a level both proximal and distal to cAMP generation. The authors hypothesize that the subsequent step may be associated with the enhancing role of calcium ions on the transport of endogenous cholesterol to mitochondria and its binding to the action site of cytochrome P-450. The inhibitory effect of calcium channel blockers verapamil, nimodipine, nifedipine and niludipine on immature granulosa cells in our studies indicates that the full responsiveness of granulosa cells to steroidogenic stimuli depends on extracellular sources of calcium. FSH, as was reported by Grasso and Reichert (1989), induced the uptake of \(^{45}\)Ca\(^{2+}\) by proteoliposomes and cultured rat Sertoli cells. In these experiments, the use of calcium channel-blocking agents reduced FSH-induced \(^{45}\)Ca\(^{2+}\) uptake to basal levels and FSH-stimulated oestradiol secretion by up to 50%. We suggest that the luteinization factor may act by a similar mechanism to increase intracellular calcium concentrations to a level optimal for steroidogenic processes by opening plasma
membrane calcium channels. In relation to this, we have studied the role of intracellular pools of calcium in mediating the action of LS.

To increase intracellular Ca\textsuperscript{2+} levels we have used Ca\textsuperscript{2+} ionophore A23187, a putative stimulator of calcium influx (Reed and Hardy 1972). A23187 decreased stimulatory activity of LFF, conditioned media and cell extracts on progesterone production and inhibited progesterone secretion induced by FSH. It was demonstrated that A23187 in the concentration range 1–5 µM stimulated basal progesterone production, but potentiated the stimulatory effect of LH on cyclic AMP and progesterone synthesis in granulosa cells when used in concentrations up to 1 µM (Veldhuis and Klase 1982, Jamaluddin et al. 1992). The inhibitory effect of A23187 in our experiments may be due to its high (2 µM) concentration which could have caused depletion of intracellular calcium. Furthermore, as was reported by Asem et al. (1992), A23187 and ionomycin in this concentration caused cytosolic alkalinization in chicken granulosa cells. The changes in intracellular pH may play a certain role in the regulation of steroidogenesis. However, the inhibitory effects of A23187 in our studies cannot be attributed to ionophore toxicity because of its stimulatory action on cGMP synthesis. Our data are in accord with earlier reports demonstrating stimulatory action of A23187 and LH on cGMP formation in rat testicular interstitial cells (Lin et al. 1981). The same stimulatory effect on cGMP synthesis was obtained with verapamil in our experiment when was applied at a lower concentration (10 µM). However, cGMP accumulation was inhibited in the presence of other calcium channel blockers nimodipine, nifedipine, niludipine and the calmodulin antagonist trifluoroperazine. These observations suggest that cGMP synthesis is dependent on extracellular Ca\textsuperscript{2+} and may be modulated by changes in intracellular calcium levels. Although gonadotropins and LFF stimulate cGMP formation in granulosa cells (Makris and Ryan 1978, Ranta et al. 1985, Kolena et al. 1993), the role of cGMP in steroidogenesis has not been clearly defined. It is suggested that cGMP may be involved in the regulation of some cellular processes as a second messenger (Garbers 1989, Hofmann et al. 1992). The effects of cGMP are mediated by the cGMP-gated calcium channel, cGMP-modulated phosphodiesterase and cGMP-dependent protein kinase (kinase G). In rat ovarian granulosa cells, dbcAMP as an activator of kinase G increased the formation of progesterone and LH receptors (Sanders and Midgley 1983). In contrast, cGMP analog did not affect P450 \textsubscript{SCC} mRNA accumulation and progesterone production in porcine granulosa cells (Urban et al. 1991). Further studies are needed to elucidate the role of cGMP in steroidogenic processes of granulosa cells.

Many of the effects of intracellular calcium are mediated by its binding to calmodulin (Cheung 1980). This Ca\textsuperscript{2+}-binding protein was demonstrated in high concentrations in porcine granulosa cells (Veldhuis et al. 1984). Our results show that calmodulin antagonist trifluoroperazine inhibited synthesis of cGMP and progesterone induced by FSH. This is consistent with reported inhibitory effects of trifluoroperazine on FSH-, LH- and GnRH-stimulated responses in granulosa cells (Veldhuis and Klase 1984, Eckstein et al. 1986, Jamaluddin et al. 1992). Furthermore, we observed inhibitory effect of trifluoroperazine on progesterone production stimulated by LFF, granulosa cells-conditioned media and granulosa cell extracts. These results indicate the possible role of calmodulin in mediating the action of luteinization stimulatory activity in granulosa cell cultures. Further experiments will be undertaken to evaluate the putative interaction of Ca\textsuperscript{2+}-calmodulin with other intracellular signalling systems in immature granulosa cells.

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References


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